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DIETARY INTERRELATIONSHIPS:
I. Anti-Anemia Factors and the Biological Methylating Agents

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DIETARY INTERRELATIONSHIPS: I. Anti-Anemia Factors and the Biological Methylating Agents

By RUTH WOODS

The recent isolation of folic acid and of vitamin B_{12} climaxed a search of more than two decades for the factors in liver responsible for the cure and prevention of the anemias. Strangely enough, the discovery of these vitamins has not marked the conclusion of a story. It has, in fact, opened up a whole series of new chapters in which it has been found that the anti-anemia factors possess many new, unsuspected functions in addition to their roles in blood regeneration. The present Review will describe one of these newer roles of the anti-anemia factors—their interrelationship with the biological methylating agents, choline, methionine and betaine.

The Concept of Biological Transmethylation:

The occurrence of biological transmethylations and the need for a dietary supply of methyl groups constituted two new concepts in metabolism when first described about ten years ago. In the years that have followed, nutrition research has advanced by geometric progression at a breath-taking pace. With the overwhelming accumulation of new findings has come, in addition, an increased insight into the meaning of existing knowledge. In many cases —of which the transmethylation concept is but one example—this new insight has made it possible to reconcile perplexing discrepancies in present knowledge and has led to new revisions and modifications of established concepts.

The Original Concept of Transmethylation: The concept of transmethylation proposed the transfer in the body of methyl groups ($—CH_3$) from one compound to another. Certain methyl groups, provided pre-formed in the diet, possessed the property of being "labile" and could thus be utilized in the metabolic process of transmethylation. It was further believed that a dietary supply of labile methyl groups was essential, inasmuch as these could not be synthesized by the body. These labile methyl groups were required to permit the synthesis in the body of important metabolites (as, for example, choline or the amino acid, methionine) from precursors existing in the diet or in the body. While the basic principles of the transmethylation reaction remain valid, the mechanisms whereby these reactions are believed to occur have been considerably elucidated and entail several modifications of the original theory. Three phases of this phenomenon merit attention in the light of new findings during the past two years:

1. The essentiality of methyl groups in the diet is a matter of degree, depending on environmental conditions and other dietary components (much as in the sense of the "essential" *vs.* "non-essential" amino acids*) inasmuch as it has been shown that the animal organism can synthesize these groups to a certain extent, but often at a rate insufficient to meet biological needs.

2. "Labile" methyl groups are transferred *in toto* in the process of transmethylation. They may also undergo degradation and resynthesis or may be synthesized from appropriate precursors under certain conditions; such newly synthesized methyl groups may then also participate *in toto* in transmethylation.

3. Methyl donors have been shown to have a high degree of specificity. In other words, compounds containing "labile" methyl groups are not all equivalent, but each methyl donor may have its own specific methyl acceptor. Moreover, each of these individual reactions is catalyzed by a specific enzyme system. Vitamin B_{12} may possibly function as part of at least one such enzyme system.

The present Review will describe some of the newer findings concerning the transmethylation process, with particular reference to the studies leading to knowledge of its biosynthesis in the animal body. The additional findings described in (2) and (3) above will be described in a subsequent issue.

The Methylating Compounds:

Most important of the compounds designated as methyl donors are *choline, betaine,* and the amino acid, *methionine.* All of these compounds have been shown to have important functions not only in the nutrition of experimental animals, but in the nutrition of poultry and humans as well.

Functions of the Methylating Compounds: In the rat these compounds possess lipotropic and hemorrhagic activities (1, 2). That is, they are necessary to prevent abnormal metabolism of fat resulting in fatty degeneration of the liver and also the condition known as hemorrhagic disease of the kidneys. These functions are largely an attribute of the vitamin, choline. The mechanism involved is not yet fully known, but it is apparently independent of the methylating capacity of choline since choline analogues which are not methyl donors (such as arsenocholine) or which have no methyl groups at all (such as triethylcholine) also are lipotropic and antihemorrhagic (3, 4).

In all species choline, methionine and related compounds are needed for growth. It was postulated that the labile methyl group of choline might be transferred to homocystine to form the essential amino acid, methionine (4, 5). Conversely, the labile methyl group of methionine might be transferred to a precursor, such as ethanolamine, to form choline (6, 7). By virtue

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**cf. Borden's Review of Nutrition Research,* November, 1950, "Dynamic Interrelationships in Amino Acid Nutrition."

of its making possible the synthesis of choline in this way, methionine could function as a substitute for choline in preventing hemorrhagic kidneys or abnormal fatty deposition in the liver.

In poultry, choline, methionine and more especially betaine are important for proper growth, for efficient egg production and for the prevention of the slipped tendon disorder known as perosis. In man, these compounds have been found to be of some value in the treatment of liver and kidney disease in addition to the basic function of methionine as an essential amino acid.

Early Studies on Biosynthesis of Methyl Compounds:

The studies leading to the investigation of the transmethylation concept as a newly recognized process in metabolism had their start in the report by du Vigneaud, Chandler, Moyer and Keppel (5) of Cornell that rats could grow on a diet free of the essential amino acid, methionine, and containing homocystine, *if choline were added to the diet.* This suggested the possibility that homocystine (or the related compound, homocysteine) was converted in the body to the essential methionine by acquiring a methyl group, presumably from choline—either directly or indirectly.

At this time, however, it was noted that for some unexplained reason an occasional animal was able to grow slowly on the methionine-free diet even if choline were not supplied. This significant exception to the general behavior of animals on this type of diet was the first clue to future findings on the mechanism of methyl group reactions. The explanation of this discrepancy could not readily become apparent until nearly ten years later when research in another field led to the isolation of vitamin B_{12}, the long-sought anti-pernicious anemia factor.

In the light of nutrition knowledge as it existed at the time, du Vigneaud and coworkers stated that this behavior might be explained by the phenomenon of refection*, or else by the operation of certain other factors of which they were not then cognizant.

Following this preliminary report, du Vigneaud *et al* further investigated the transmethylation hypothesis. In an extensive series of researches using isotopic tracers to follow the metabolic fate of the materials under investigation, they clearly demonstrated the existence of transmethylation processes in the rat and later, in man as well.**

Mechanisms of Biological Methylations: Some four years after the first report of du Vigneaud's group, further unusual findings were reported by Toen-

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*Synthesis of vitamins and other nutritional factors by intestinal microorganisms.

***cf. Borden's Review of Nutrition Research,* October, 1942, "Labile Methyl Groups of Choline and Protein."

nies, Bennett and Medes (8) at the Lankenau laboratories in Philadelphia. They found that a large number of their animals consistently grew on a stock diet containing homocystine, but no methionine or choline. This was in marked contrast to the finding of the Cornell group that only an occasional animal was able to grow on such a diet. (Growth of animals on a diet low in methyl groups or even entirely free of them had also been reported on occasion by other investigators. Brand (9) had noted some growth of animals on a diet containing homocystine supplemented by milk and yeast concentrates. Similarly, White and Beach (10) had obtained growth of animals with homocystine when the diet was supplemented with ryzamin B* and liver extract as did Rose and Rice (10a) using milk concentrate and tikitiki as a source of vitamins.)

Bennett, Medes and Toennies (11) investigated this phenomenon in considerable detail and, as a result, set forth several provocative speculations. Chief among these was (1) the theory that the animals might be furnished with labile methyl groups originating from bacterial synthesis in the intestine as originally suggested by du Vigneaud and coworkers or (2) there might be vitamin factors, of either dietary or intestinal origin, the presence of which might enable the animal to compensate for the absence of dietary methyl donors by biosynthetic means of its own or of its intestinal bacteria. In connection with the latter suggestion, Bennett *et al* postulated a possible effect of the pre-experimental diet, which differed from that used at Cornell, on the subsequent biosynthetic activity of the intestinal flora.

In a further study, these workers made analyses of rat tissues and were able to demonstrate the actual synthesis of a small amount of the essential amino acid, methionine, in rats on a diet containing homocystine and no dietary source of labile methyl groups (12). The synthesis of labile methyl groups, to a degree, was also demonstrated by du Vigneaud's group through studies with isotopically labelled water (13).**

Later Investigations of Methyl Group Biosynthesis:

At this point, the Lankenau group and the Cornell group got together in an attempt to further explore the unusual nature of these findings, Bennett and coworkers then repeated their studies with a group of rats obtained from du Vigneaud's laboratory. These animals were kept on the Lankenau

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*A rice polishings extract providing B vitamins.

**These animals were maintained on a diet adequate in methyl groups. Nevertheless, biosynthesis of methyl groups also occurred. Evidence for this was obtained by maintaining the deuterium concentration of the body water of the rats at about 3 atom percent and subsequently isolating the choline from the tissues. The occurrence of deuterium in the methyl groups of choline was indicative of the biosynthesis of these methyl groups. Whether such synthesis occurred in the intestinal tract or in the tissues was not possible to determine at this point.

stock diet and animals bred from this strain were then used in studies on the utilization of homocystine on diets free of choline and methionine. It was found that these animals also were capable of using homocystine under these conditions, although somewhat less effectively than did the animals of the Lankenau laboratory. This strongly suggested that pre-experimental nutrition, and not strain differences, was a determining factor in explaining the behavior of both groups of animals.

Bennett *et al* (15) suggested that their unusually nutritious pre-experimental diet could act in one or both of the following ways: (1) by promoting nutritional storage in the animal body of factors required for subsequent assimilation of homocystine and (2) by enhancing development of its intestinal organisms which in turn become the actual contributors of the essential factors.

Studies on Effect of Intestinal Synthesis: In order to examine the second of these possibilities, studies were made in which sulfasuxidine* was incorporated into the diet containing homocystine (16). This sulfonamide is known to inhibit the growth of intestinal bacteria and, as a result, the synthesis of various vitamin factors, notably folic acid and biotin (17). On this diet, growth of the animals was interrupted in about four weeks. Addition of a folic acid concentrate (not present in the 8 vitamin supplement used in the diet) failed to restore growth, thus indicating that a disturbance in the intestinal synthesis of folic acid was not the cause of the growth failure. Similarly, the provision of extra biotin (in addition to that present in the 8-vitamin diet supplement) failed to have any effect. When a small amount of choline or of a liver fraction was added, the animals on the sulfonamide diet were able to use homocystine. Bennett *et al* concluded that the liver fraction seemed to take the place of an intestinal factor eliminated by the sulfonamide action. The activity of the liver fraction was shown *not* to be due to its minute content of choline, methionine or betaine, the response being entirely out of proportion to the trace amounts of methyl donors present. It seemed at the time, that the anti-pernicious anemia principle was not the effective component of the liver fraction (15), since addition of this fraction alone failed to permit growth with homocystine.

The Role of Folic Acid: Some time after these studies, crystalline folic acid became available and this vitamin was, therefore, added to the original supplement of eight crystalline vitamins. Wide variations in the behavior of the animals was observed. In some, crystalline folic acid in large doses enabled the animals to continue growing on the homocystine-sulfonamide diet; in others, extra folic acid failed to counteract the growth-inhibiting

*Succinyl-sulfathiazole.

effect of the sulfonamide, these animals responding, however, to the liver extract. Bennett (18) commented,

> "The fact that only some rats respond to folic acid indicates that it is not the only factor involved. These animals retain a latent capacity for the utilization of homocystine which seems to depend for its manifestation on a fairly high level of folic acid and is not destroyed by the sulfa drug. The nature of the variability of the growth response becomes apparent when we realize that other vitamin B factors are probably involved, the most conspicuous one being B_{12}. These factors may be stored in the animals in varying amounts from pre-experimental diets and may therefore be depleted in single animals at different periods of the experiment."

In subsequent reports (25, 25a) Bennett confirmed this suggestion by showing that when growth had leveled on a "labile methyl" free diet containing adequate amounts of folic acid, addition of vitamin B_{12} promoted growth.

By this time, evidence was beginning to point again in the direction of the anti-anemia factors—folic acid and vitamin B_{12}, both of which had only recently been identified and isolated—as the agents probably involved in the biosynthesis of essential methylated compounds.

Evidence along these lines was further substantiated by a host of other findings in the field of poultry nutrition. These researches, in an entirely different field, similarly led to the recognition of an interdependence between the methylating agents (methionine, choline, betaine) and the newly recognized "animal protein factor" (eventually identified as vitamin B_{12}).

Animal Protein Factor and Methylating Compounds:

Long before the existence of the animal protein factor was conclusively established, many investigators in poultry nutrition had made the interesting observation that methionine is effective in improving the nutritional value of both raw and heated soybean meal.* This was the first in the chain of events which eventually were to link the animal protein factor to the phenomenon of transmethylation.

Early Studies with Methionine: In 1947, Bird and coworkers (19) showed that the addition of methionine to a high soybean diet produced as good growth in chicks as did the animal protein factor provided, however, that this factor had been present in the diet of the mother hens. Methionine was less effective when fed to chicks from hens whose diets had been deficient in the animal protein factor. Inasmuch as the basal diet provided adequate amounts of the essential amino acid, methionine, the reason for the activity of the extra methionine was not clear although it was clearly

*cf. *Borden's Review of Nutrition Research,* June, 1944, "The Nutritive Value of Soybeans."

indicated that some interrelation of the amino acid with the new vitamin existed.

Rubin and Bird (20) suggested three possible explanations: (1) the growth factor might facilitate the liberation of methionine from soybean protein during digestion; (2) the growth factor might function in the metabolism of methionine after absorption; or (3) it might have some entirely different metabolic function which could be partially performed by methionine. On the basis of other studies, by Cary and Hartmann (21) for example, another explanation was suggested, i.e. simply that the need for the animal protein factor might be intensified as the quantity of protein (as, soybean meal) is increased in the diet.

Recent Studies: In May, 1949, Gillis and Norris (22) presented more definite conclusions for the belief that an interrelation exists between the animal protein factor and the methylating compounds important in poultry nutrition. They pointed out that the choline requirement of chicks fed a purified diet is generally regarded as approximately 0.1 per cent of the diet. With practical diets, however, they continue, more rapid growth is usually obtained when betaine, choline or other methlyating compounds considerably in excess of this requirement are provided. Since such diets, unsupplemented, would not ordinarily be considered deficient in methyl groups, these workers proposed to determine the factors which might influence the need for biologically available methyl groups.

They, therefore, conducted a series of experiments with both normal chicks and with chicks partially depleted in the animal protein factor (APF). A diet complete in all known vitamins and composed of mixed cereals, plant protein and supplementary minerals and vitamins was found to be significantly improved—for both normal and APF-depleted chicks—by additions of betaine or choline (Table 1).

Since betaine was as effective as choline, it was believed that the effect was probably due to the increased supply of labile methyl groups. However, when the liver paste (source of animal protein factor) was added, the growth effect was considerably superior to that obtained with the methylating agents alone. Furthermore, when the liver paste was included, extra betaine or choline caused no further improvement. The choline content of this particular liver was negligible (0.0018%); its vitamin B_{12} activity was approximately 17 micrograms per gram.

These studies showed that a source of animal protein factor or of vitamin B_{12} may decrease the need for supplementary methylating compounds. This was interpreted to mean that at least one metabolic function of the animal protein factor is concerned with transmethylation. The authors suggested

Table 1.

Supplement	Weight, end of 3 wks. depletion period*	Gain in weight, 4 to 7 wks. inclusive†
	gm.	gm.
None	146	248
0.2% betaine. HCl	148	339
0.2% choline. HCl	144	335
0.15% liver paste‡	144	450
0.15% liver paste + 0.2% betaine. HCl	149	454
0.15% liver paste + 0.2% choline. HCl	147	445

*During the first 3 weeks all the chicks received the unsupplemented diet; i.e. completely adequate except for a source of the animal protein factor.

†Nineteen white Leghorn male chicks were used in each lot; no mortality occurred during the 4–7 week period.

‡A commercial liver paste found to supply the chick's requirement for APF in the amount given.

that either transmethylation is more efficient in the presence of an adequate amount of APF or that a partial deficiency of APF creates or stimulates metabolic processes which require excess methyl groups.

Almost simultaneously with the report by Gillis and Norris, their findings were confirmed as a result of independent researches by Schaefer, Salmon and Strength (23, 24). In view of the relationship between methionine and choline, Schaefer and coworkers decided to determine whether vitamin B_{12} might exert a sparing action on choline.

Their studies were conducted with rats (23) and were designed to determine the effect of supplementing a low choline-methionine diet with vitamin B_{12} in concentrate or crystalline form. They found that both the incidence and severity of kidney damage in rats on such deficient diets were markedly decreased by supplementing the diet with vitamin B_{12}, either as a concentrate or in crystalline form. They found that 30 micrograms of vitamin B_{12} per kilo of diet could replace as much as one-half of the supplementary choline or methionine required for protection against kidney damage. The addition of the vitamin, under these conditions, also caused a significant gain in weight which did not occur when the vitamin was added in the presence of adequate levels of choline.

These workers also investigated the interrelationship between vitamin B_{12} and choline with respect to the nutrition of the chick (24). A similar interrelation was found in that supplements of vitamin B_{12} diminished to an appreciable extent the amount of choline required for optimum chick growth.

As pointed out by Gillis and Norris (22), however, vitamin B_{12}* apparently performs other functions necessary for chick growth because the response obtained with this vitamin (in their studies as well as in those of Schaefer and coworkers) was much greater than that which was obtained with methylating agents alone.

By this time Bennett had confirmed her earlier suggestion (18) of the role of vitamin B_{12} as a factor in the biosynthesis of methyl groups. She reported that vitamin B_{12} plus folic acid gave an effect similar to that which had been obtained previously with the crude anti-pernicious anemia liver extract in rats on methyl-free diets containing homocystine (25). The need for *both* folic acid and vitamin B_{12} explained both the partial response which had been obtained with folic acid alone, and the apparent ineffectiveness of the liver extract used in conjunction with an eight-vitamin supplement which did not include folic acid.

Coincident with Bennett's report, similar findings were announced by other investigators. Thus Jukes and Stokstad (26, 27) obtained evidence indicating that vitamin B_{12} is involved in the methylation of homocystine to methionine in the chick. In a soybean meal diet, adequate in vitamins and minerals, growth responses were obtained with either homocystine or methionine if vitamin B_{12} was also present in the diet. In the absence of vitamin B_{12} only methionine gave a response, while homocystine was completely ineffective.

Very similar findings were reported by Stekol and associates (28, 29). They found that rats are able to grow on a diet free of all the known labile methyl group donors but which contained vitamin B_{12} and homocystine or homocysteine. The test diet was complete with respect to all essential amino acids, except methionine, and it contained all the known vitamins including folic acid and biotin. Further, on a diet containing ample methionine (as the sole sulfur-containing amino acid), but no vitamin B_{12}, growth was poor. Growth was good when B_{12} was added to the methionine-containing diet. Of considerable significance was the fact that none of the experimental animals receiving vitamin B_{12} died of kidney lesions in spite of the complete absence of labile methyl group donors in the diet. These workers, however, pointed out that young rats (under 30 days) are unable to survive on a diet free of methyl donors, presumably because biosynthesis does not proceed at a rate rapid enough to meet the heightened demand of young animals.

The precise mechanisms involved in the B_{12}-folic acid-methyl synthesis story are still a matter of active investigation.* It is interesting to note that

*This was a study conducted with pure vitamin B_{12} which confirmed the belief that this vitamin was responsible for the activity earlier reported for the APF concentrate.

the choline content of the various diets ranged from 0.2 gram to 0.6 gram per 100 grams of diet, whereas the B_{12} equivalent of these quantities of choline was from 1 to 3 micrograms of B_{12} per 100 grams. Thus the amount of B_{12} was the order of 1/100,000 of the physiologically equivalent amount of choline. This suggests that vitamin B_{12} might function as an enzyme releasing methyl groups from methylated compounds in which the methyl group is not ordinarily labile and/or that B_{12} may function as part of an enzyme system involved in the *synthesis* of methyl groups in the body.

Further evidence for a mechanism of the first type seems to be indicated in very recent studies by Schaefer, Salmon and Strength (30). They noted that the effectiveness of methyl donors such as monomethylaminoethanol (one methyl group), dimethylaminoethanol (two methyl groups), betaine, methionine and choline (trimethylaminoethanol) was considerably improved in the presence of vitamin B_{12}** with respect to the amounts required for prevention of renal damage and fatty livers in rats and growth and prevention of perosis in chicks. Further, on the basis of methyl content, monomethylaminoethanol and betaine are considerably less effective than dimethylaminoethanol and choline in the prevention of liver and kidney disorders in the rat. When vitamin B_{12}* was added, all these compounds became equivalent to choline on a methyl basis. The presence of vitamin B_{12} appeared to be necessary for the effective use of these compounds in chick nutrition as well. In these studies it would appear, therefore, that vitamin B_{12} might act to increase the lability of the methyl groups in methylated compounds which are less effective, on a methyl content basis, than choline. The possibility, previously mentioned, that new sources of labile methyl are created from unknown compounds having methyl groups usually regarded as non-labile, is not excluded as an additional hypothetical explanation of the action of vitamin B_{12} in these experiments.

The studies by Schaefer *et al* (30) also support the belief, as suggested by Bennett, that folic acid may be involved, along with vitamin B_{12}, in the methylation process. This fact was still further emphasized in a report by Salmon (31) in which studies with rats indicated that the utilization of methionine is significantly affected by the protein level of the diet (excessively high protein increasing the requirement for methionine) as well as by the presence of vitamin B_{12} in combination with folic acid.

Conclusion:

An important new role has been discovered for the anti-anemia factor,

*This will be discussed further in the next issue of BRNR.
**Plus folic acid (folacin).

vitamin B_{12}. This is its as yet unexplained relation to the process of transmethylation in the rat and in the chick. Although this is an activity apparently quite distinct from its role in blood formation and regeneration it is a curious fact that another anti-anemia compound, folic acid, also seems to be involved in the transmethylation activity of vitamin B_{12}. Because of the unique interrelation of the anti-anemia factor with choline and other biological methylating agents, it has been suggested (23) that choline be tried as a supplementary aid to B_{12} and folic acid in the clinical treatment of various types of human anemias, particularly in cases complicated by liver damage.

Although the mechanisms involved in these activities are still obscure and the mysteries of the intermediary metabolism of vitamin B_{12} are still not completely solved, these findings have already found practical application in the construction of better rations for domestic animals, poultry and swine. One may speculate endlessly on the many pathways which may be opened up by the application of these principles, when they are finally elucidated, to problems of human physiology, but positive conclusions must await further research in this intriguing field of study.

The next issue of BORDEN'S REVIEW OF NUTRITION RESEARCH will describe more recent findings concerning the nature and significance of the biosynthesis of methyl groups, as well as studies with germ-free animals and possible pathways involved in the intermediate metabolism of methyl groups.

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